FISH on Biofilms from flow chambers:

Fixation of biofilms

Fixative (4% pareformaldehyde) is prepared and kept on ice until use. Warning - Paraformaldehyde is very carcinogenic - use gloves and hood!

- Stop the pump and clamp off the inlet on those channels that need to be fixed.
- Remove the channels from the pump and start the pump again.
- The flow channel is then filled with ice-cold fixative by carefully injecting 0,5 mL with a syringe as close as possible to the inlet.
- Adding a thin layer of silicone rubber glue seals the injection hole.
- Incubate for 1 hour (or over night in an ice box in the fridge to keep cold) - preserves unstable Gfp.

Embedding of biofilms

- Bring the flow chamber and effluent tubing to a flow bench.
- Put the effluent tubing into an Ole Dich 4 channel pump and take off the clamp.
- Put 1xPBS into a small beaker, and then wash the biofilm for 10 min., at a pumping rate of 180 μL/min. For careful treatment, wash for 20 min. at a rate of 90 μL/min.

The biofilm is now ready for embedding.

For embedding of two channels mix following in a 2 mL Eppendorf-tube:

- 1,0 mL 20 % Acrylamide (Warning, Acrylamide is a strong nerve-toxin, use gloves and hood).
- TEMED 8 μL

A number of Eppendorf-tubes can be prepared before embedding starts.

- Start by reversing the pump flow until there is a small drop at the end of each tube, and make sure that the inlet tubing is free of bubbles.
- Turn the direction of the pump flow again (Pump speed set to 180 μL/min).
- Add 16-20 μL of 1 % APS to the Acrylamide solution and invert the tube a couple of times. Do **not** shake as this will introduce more oxygen into the solution which can inhibit polymerization. (IMPORTANT: the amount of APS added determines the time for the Acrylamide solution to polymerize, therefore make a test in an Eppendorf tube before starting the embedding procedure on the flow channel biofilms. The polymerization time should be about 3-5 min.).
- Put the tubing from the flow channels down into the Eppendorf tube with the Acrylamide solution.
- Pump for app. 2 min. drawing the solution into the channels at a speed of 180 μL/min, while holding the flow cell vertical.
- Immediately after clamp off the effluent tubing and remove it from the pump.
- Let the Acrylamide solution solidify for 1 hour.

- Put the flow chamber with the Acrylamide embedded biofilm in a humidified petri dish sealed with parafilm and store at 4°C until use (If there is Gfp in the sample the process should be continued).

Hints:
- Use nitril gloves.
- Use a 2 mL Eppendorf tube.
- Make sure that the inlet tubes are equally long before embedding, and that they reach the bottom of the Eppendorf-tube – but not too long (ca 5-7 cm).
- The polymerization of the Acrylamide solution is inhibited by oxygen therefore mix gently.

Removing embedded biofilms from the flow channels

The glass between each channel is cut with a diamond glasscutter. Each piece of glass is taken off by loosening the glass from the Plexiglass with a scalpel. Be careful not to touch the Acrylamide block. BE CAREFUL: cut away from yourself and your fingers!. Start by loosening and lifting up at the end of the channels and then at the sides.

The glass will break into pieces but will be connected due to the silicone rubber. Remove the glass completely from the Acrylamide block.

Add a few drops of Milli-Q water to the Acrylamide block and carefully loosen the ends of the block with a scalpel. Then slide the entire Acrylamide block unto the scalpel and place it on an object glass and cover it with 1 mL Milli-Q water. Make sure that the side that faced the glass cover slip in the flow cell is turned downward!

Put object glass plus biofilm block into a petri dish, take a piece of wet tissue and put it at the side. Seal the petri dish with parafilm, store at 4 °C.

Hints:
- Remember to note the direction of flow.
- After use the flow cells should be cleaned in ethanol, but only for a minute to avoid cracks in the Plexiglass.

Hybridization of embedded biofilms

- Cut out a block from the embedded biofilm of approximately 5 mm.
- Put the small biofilm block on an object glass with wells as shown in figure 3. Make sure that the side that faced the glass cover slip in the flow channel is turned downward.
- Add 45 μL of Washing solution I (with 30 % Formamide).
- Pre-hybridize at 37 °C for 30 min. in a humid chamber, see figure 3. Hint: wet some tissue paper with Wash I and place it in the chamber together with the slide,
- In the mean time find the probes and prepare the hybridization solution. The probes should always be thawed and kept on ice in the dark. For one piece of biofilm mix probes and Washing solution I (30 % FA) to a final volume of 30 μL. The concentration of the probes should be app. 2.5 ng/μL. Mix solution gently.

- Remove the pre-hybridization solution with a Gilson pipette.
- Add the hybridization solution (30 µL/well).
- Incubate at 37 °C for at least 2,5 hours (max. over night).
- Remove the hybridization solution.
- Add 45 µL of Washing solution I (pre-warmed to 37 °C), pipette a few times up and down with a Gilson. Remove the solution.
- Add 45 µL of Washing solution I again.
- Incubate at 37 °C for 30 min.
- Remove Washing solution I.
- Add 45 µL of Washing solution II (pre-warmed to 37 °C), pipette a few times up and down with a Gilson. Remove the solution.
- Add 45 µL of Washing solution II again and incubate at 37 °C for 30 min.

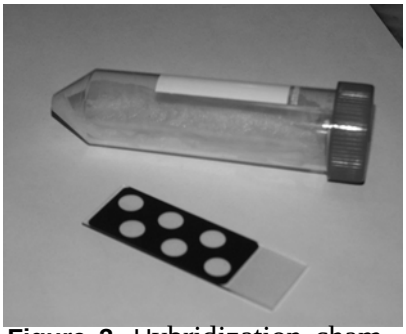

Figure 3. Hybridization chamber and an object glass with wells.

Prepare an object glass with a silicone rubber packing (24 mm x 50 mm x 1 mm) on top. Cut a hole in the silicone rubber where the piece of hybridised biofilm can fit.

Have the following ready: Object glass, 24 mm x 50 mm x 1 mm cover slip, antifade solution (use Slowfade® Antifade –Kit from Molecular probes (Component B)) and white insulation tape.

- Remove Washing solution II.
- Rinse with Milli-Q water by pipetting a few times up and down with a Gilson. Remove the water immediately after.
- Put the piece of biofilm into the hole of the pre-prepared silicone rubber packing on an object glass. Make sure that the side that faced the glass cover slip in the flow channel is turned upward.
- Add one drop of Antifade solution (component B) on top of the biofilm block.
- Put a 24 mm x 50 mm cover slip on top.
- Tape the cover slip, silicone rubber and object glass together (use the white tape).
- Store the sample in a humidified petri dish sealed with parafilm dark and at 4 °C.

Hints:
- Warning - Formamide is carcinogenic - always work with nitril-gloves in a hood.
- If there is Gfp, the sample should preferably be visualized immediately after it is prepared.